DIRECT IMMUNOFLUORESCENCE STUDY OF 25 CASES OF SUBEPIDERMAL BLISTERS

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CERTIFICATE

This is to certify that this dissertation entitled **"DIRECT IMMUNOFLUORESCENCE STUDY OF 25 CASES OF SUBEPIDERMAL BLISTERS"** is a bonafide work done by **Dr.VIJAY SINGHAL,** Post Graduate in M.D. Dermatology, Venereology and Leprosy, Madras Medical College, Chennai- 600 003, during the academic year 2006-2009. This work has not been formed previously the basis for the award of any degree.

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DECLARATION

I, **DR.VIJAY SINGHAL**, solemnly declare that this dissertation titled **"DIRECT IMMUNOFLUORESCENCE STUDY OF 25 CASES OF SUBEPIDERMAL BLISTERS"** is a bonafide work done by me at Madras Medical College during 2006-2008 under the guidance and supervision of **Prof. Dr. B. Parveen, M.D., D.D.,** Professor and Head, Department of Dermatology, Madras Medical College, Chennai - 600 003.

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CONTENTS

INTRODUCTION

Several skin diseases are characterized by the presence of blisters. In some, this may be the only morphologic clue to the diagnosis, whereas in other disease, blisters may be one of the several manifestations.

The immunobullous disorders are a group of autoimmune diseases in which components of the epidermis and basement membrane zone are the focus of attack, resulting in the formation of cutaneous and mucosal blisters. The autoimmune bullous diseases result from an immune response to molecular components of intercellular adhesion molecules in desmosomes or the basement membrane zone (BMZ). The diagnosis of autoimmune bullous diseases is based on the evaluation of clinical findings, histopathology, direct immunofluorescence (DIF), and indirect immunofluorescence (IIF). The autoimmune blistering diseases may be subdivided into intraepidermal (pemphigus) and subepidermal (pemphigoid) blistering disorders on the basis of the level at which blistering occurs.

Prior to the early 1950s, most generalized bullous diseases were considered to be variants of pemphigus, regardless of the level of skin cleavage or differences in clinical appearance or course. Pemphigus and

pemphigoid, the two most common autoimmune bullous disorders, were first well separated by Lever in the early 1950s[1]

With the development and application of immunofluorescence microscopy in the mid 1960s further distinctions were made not only between pemphigus and pemphigoid but among other autoimmune diseases also. Immunofluorescence testing is of utmost value in confirming a diagnosis that is suspected by clinical or histologic examination. This is especially true in subepidermal bullous diseases that often have overlap in the clinical and histologic findings.

REVIEW OF LITERATURE

A blister is a fluid filled cavity formed with in or beneath the epidermis. The fluid consists of tissue fluid, plasma and variable amount of inflammatory cells. Artificial distinction has been made into small (vesicles, <0.5cm) and large blisters (bullae,>0.5cm). Blisters in the skin can occur at any age and may be caused by common infections, rare genetic skin diseases or auto immunobullous diseases.

Depending on the level of blister in the skin, immunobullous diseases can be classified into intraepidermal or subepidermal.

Intraepidermal bullous disorders include:

Pemphigus vulgaris

Variant: pemphigus vegetans

Pemphigus foliaceous

Variant: pemphigus herpetiformis

Variant: pemphigus erythematosus

Intercellular IgA dermatosis

Paraneoplastic pemphigus (PNP)

Subepidermal bullous disorders include:

Bullous pemphigoid

Variant: pemphigoid nodularis

Variant: pemphigoid vegetans

Variant: lichen planus pemphigoides

Mucous membrane pemphigoid

Pemphigoid gestationis

Linear IgA disease

Variant: chronic bullous disease of childhood

Variant: linear IgA disease of adults

Epidermolysis bullosa acquisita

Bullous systemic lupus erythematosus

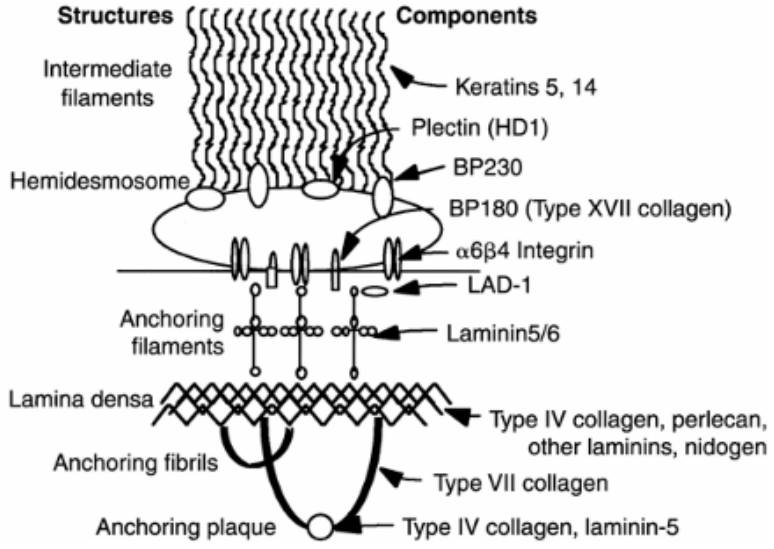
Dermatitis herpetiformis

Subepidermal autoimmune blistering disorders arise due to antibodies targeting various antigens located in the basement membrane zone (BMZ).

Structure Of Skin Basement Membrane Zone (BMZ) / Dermoepidermal Junction (DEJ)

Skin basement membrane zone (BMZ) is an ultrastructurally defined area situated between the epidermis and the dermis. Skin BMZ is 0.5-1.0 mm-thick band-like structure that is positively stained by periodic acid-Schiff (PAS) stain. The major function of skin BMZ is to serve as an adherent connection between the epidermis and the dermis. The skin BMZ can be divided into four ultrastructurally distinct areas:

1. Hemidesmosome
2. Lamina lucida
3. Lamina densa
4. Sub-lamina densa.



STRUCTURE OF BASEMENT MEMBRANE ZONE

Hemidesmosome

It contains BP Ag1 and 2, α6β4 integrin and plectin. The BPAg1 (BP 230) is located intracellularly whereas the BPAg2 (BP180; collagenXVII) is a transmembranous protein that contains an intracellular domain, a transmembranous segment, and an extracellular domain that projects into the lamina lucida. The extracellular domain of BP180 protein contains a collagenous domain interrupted by 16 small non-collagenous domains. The largest of these 16 non-collagenous domains, NC16A, is located adjacent to the transmembranous segment. In addition, a member of the integrin family, α6β4, and plectin (a cytoskeleton-associated attachment protein), are also located in this area. Anchoring filaments originate at hemidesmosomes and insert into the lamina densa.

Lamina lucida

Situated between the hemidesmosome and the lamina densa, the lamina lucida is electron-lucent under electron microscope. Nevertheless, fine filamentous structures are observed in this area and are termed anchoring filaments. Anchoring filaments extend from the basal keratinocyte hemidesmosomes to the lamina densa, thus traversing the lamina lucida. One of the anchoring filament components is a member of the laminin family; laminin-5 (kalinin, epiligrin, nicein,

BM600).Other lamina lucida located antigens include entactin/nidogen and fibronectin. The laminin family consists of a group of heterotrimers of various combinations of three chains,α, β and γ, and are synthesized and secreted by keratinocytes. The lamina lucida appears to be the weakest zone of the DEJ. It separates easily with heat and suction, with treatment with salt solutions and proteolytic enzymes, and in diseases.

Lamina densa

Lamina densa, named according to its electron dense appearance under electron microscope, is 35-45 nm-thick. The BMZ components that are located in this area include: type IV collagen, perlecan (heparan sulphate proteoglycan), and laminin-6.Type IV collagen is considered to be the major component in this area.

Sub-lamina densa

Below the lamina densa, there are fibrillar structures that connect the lamina densa onto the dermal plaque-like structures. These fibrillar structures have been named anchoring fibrils. Type VII collagen is the major component of the anchoring fibrils. Type VII collagen is a 290-kDa protein synthesized and secreted by both keratinocytes and fibroblasts. Other minor fibers that connect to the area beneath lamina densa are: oxytalan, elaunin, and elastic fibers.

Mucosal BMZ also contain identical components as skin BMZ. Different antigens have been discovered in the four zones of the BMZ, which are the targets for various diseases. Autoantibodies targeting skin and/or mucosal BMZ components, like genetic mutations of BMZ components, result in histopathologically defined subepidermal blistering diseases. Regardless of which BMZ antigen is targeted by autoantibodies, the histopathological findings of all autoimmune subepidermal blistering diseases are similar. Although the inflammatory cell infiltrate may provide clues in the diagnosis of certain diseases, such as eosinophilic infiltrate in the diagnosis of BP, they are not always reliable and cannot be used as definitive diagnostic criteria. Therefore, more specific techniques are required for accurate diagnosis like direct and indirect immunofluorescence.

IMMUNOFLUORESCENCE

Immunofluorescence is a laboratory technique for demonstrating the presence of antibodies in tissues or body fluids. The use of immunofluorescence in both diagnosis and research is well recognised in dermatology, dating from the description of granular deposits of IgG and C3 along the dermoepidermal junction in the lesions of lupus erythematosus in 1963.[2] Beutner and Jordon (1964), used an IIF

technique to demonstrate what was then considered to be intercellular substance of stratified squamous epithelium in the serum of patients of pemphigus.[3] Later, in 1967 Beutner and Jordon described the immunological basis of pemphigoid.

Historically, Coons et al were the first group to work on the use of chemically labeled antibodies as reagents for the detection of antigens in mammalian tissue. The same authors in 1942 demonstrated the pneumococcal antigen in murine tissue by using this technique.[4]They improved the method using fluorescein as the reagent because of the brilliance of its fluorescence and the wavelength of its emitted light close to the maximum sensitivity of the human retina. In 1963 a report of dermoepidermal fluorescent band in the lesion of lupus erythematosus was published.[2]

A year later Beutner demonstrated an intercellular substance in stratified squamous epithelium using IIF technique. Since then there have been many refinements and improvements in the techniques.

The next decade saw the use of immunoelectron microscope (IEM) in understanding vesicobullous lesions of the skin. Subsequently during the 1980's western blotting, immunoprecipitation and cell culture were brought into use to study the molecular components of skin which

are targeted by the autoimmune phenomenon in various bullous disorders.

IMMUNOFLUORESCENCE TECHNIQUES

Specimen processing

DIF is performed on normal-appearing skin immediately adjacent to a lesion (perilesional skin). If the patient does not have blisters, the specimen may be obtained from skin adjacent to erythematous or urticarial plaques because the immune deposits are partially or completely degraded in inflamed or blistered skin, and DIF may be falsely negative. For IIF, blood, blister fluid or urine may be used.

Skin specimen usually obtained by using a punch biopsy or surgical biopsy from the perilesional skin. Then it is immediately snap frozen for best preservation of the tissue which is done with liquid nitrogen and maintained at -80°C. If delay is inevitable due to want of transportation, the specimen is placed in a special transport medium like Michel's medium without loss of antigenic expression.[5]

Michel's medium consists of liquid fixative in the form of ammonium sulphate which prevents degradation of tissues with immune reactants. Use of transport medium maintains a neutral pH; this is best achieved by prior washing the biopsy specimen in normal saline or

phosphate buffered saline (PBS) to remove blood and serum proteins. The biopsy specimen stays in this medium for up to 8 weeks without the loss of specific fluorescence.[6]

After the biopsy specimen is received at the laboratory liquid fixative if any is washed off in neutral buffer. It is then snap frozen and stored at-70°C to -20°C .The method of freezing varies from place to place. (Table 1). This can be done using liquid nitrogen or slow freeze in cryostat.

Sections are obtained of the unfixed frozen specimen by cutting in a cryostat equipped with an anti role device to correct the tendency of the specimen to curl. Four to six microns thick sections are cut and four sections are placed on each slide by bringing it close to the knife surface so that the sections thaw and become firmly bound to the glass. The slides are then air dried to prevent detachment during staining.

Table 1. Methods for freezing skin biopsy specimen

Method	Technique	Remarks
Liquid Nitrogen	Specimen is wrapped in aluminium foil or placed in a cryotube and then immersed into liquid nitrogen	These methods may induce freezing artifact, causing distortion of the tissue architecture
Cryospray	Specimen is placed on to a cryochuck, covered with OCT(optimal cutting temperature) compound and frozen with cryospray	
Cold Hexane	Involve the use of n hexane which is maintained at -60° C in a low temperature histo bath	This method greatly reduces the degree of artifact and allows the biopsy to be properly oriented.

Sections on each slide are incubated with FITC labeled antisera against IgG, IgA, IgM, C3, and fibrinogen. Each reagent is usually incubated for 30 minutes. The non specifically bound reactants are removed by washing the slides in a bath of PBS (pH 7.4). Washing should be carried out with all reagents for a minimum of thirty minutes. Finally stained preparations are mounted in buffered glycerol and examined with a fluorescent microscope.[7]Provided the sections are stored in the dark at 4°C; the IF is retained for 48-72 hours.

Principles of Immunofluorescence [8, 9]

Fluorescent techniques involve the emission of light of one wavelength/colour from a substance being irradiated with the light of different wavelength, the emitted light showing a low energy level. The fluorochromes used routinely in dermatopathology are fluorescein isothiocynate (FITC) and tetra methyl rhodamine isothiocynate (TRITC). FITC has an emission wavelength of 525nm and a maximum absorption wavelength of 495 nm. It emits apple-green fluorescence. TRITC has an emission wavelength of 580nm and a maximum absorption wavelength of 555nm. It emits orange-red colour.

Spectrofluorometric characteristics

Substrate	Absorption maximum	Emission maximum
FITC conjugate	495nm (blue)	525nm (apple-green)
TRITC conjugate	555nm (green)	580nm (orange-red)

To visualize the characteristic emission of the dye, different excitation and barrier filters must be used. The excitation or primary filter is placed between the light source and the tissue section to screen out the wavelengths other than those near the excitation maximum of the dye and the barrier filter removes all but the emitted wavelength. Fluorochrome conjugated antibody preparations are commercially available. These have to be stored in dark containers frozen at -200°C.

The classical fluorescent microscope has a mercury vapour lamp that generates a light beam which is reflected by a concave mirror. This is projected through collecting lenses to the excitation filter which emits a fluorescent light beam. The beam then passes through a condenser on to the specimen with the help of a reflecting mirror. This emitted light passes through a barrier filter. The resultant pattern of fluorescence is then visualized and magnified by the objective and the ocular lenses.

There are three basic types of IF used in dermatology:

Direct Immunofluorescence (DIF)

It is a one-step procedure used to detect and localize immunoreactants deposited in vivo in the patient's skin or mucosa. The immunoreactants include antibodies, complement components and fibrinogen. The direct test for the tissue fixed antibody uses a frozen section of patient’s skin on which is overlaid fluorescein or rhodamine conjugated antibody to immunoglobulins and complements. If the tissue contains an antibody e.g., IgG, then fluorescein labeled antihuman IgG will bind to it when the frozen tissue section is overlaid with a solution containing this reagent. Under fluorescent microscope the fluorescein attached to antibody is activated and emits an apple green colour. The degree of fluorescence (weak, moderate, strong) can only be approximated by the microscopist. Antibody titre cannot be determined by direct IF.

Indirect Immunofluorescence (IIF)

It is a two-step procedure used to identify circulating auto antibodies to cutaneous or mucosal structures in a patient's serum. These antibodies are most commonly of IgG or IgA classes. Indirect IF is a serological test for antibodies in serum or other fluids. The substrates

used in the detection of circulating antibodies in bullous diseases include human skin,[10]monkey esophagus,[11] guinea pig lip or esophagus, and salt-split human skin.[12] The sensitivity and specificity of the substrates may vary for the various bullous diseases.[13] Frozen tissue section or cell suspensions of this normal tissue are placed on microscope slides. Sections are overlaid with appropriate dilutions of serum or other body fluids so that any antibody against normal tissue components will bind to them. The final steps of indirect IF are identical to DIF. Fluorescein labeled antihuman immunoglobulin or complement is overlaid on the tissue section. Sections are washed and examined under fluorescent microscope. Titers of antibodies in fluids can be determined by IIF. The titer is the last dilution of serum where antibody can be detected.

SUBSTRATES USED IN IIF

Antibody type	**Substrates**
Intercellular	Normal skin Monkey or guinea pig oesophagus Rat bladder
Anti-BMZ	Monkey oesophagus Rabbit oesophagus/lip Guinea pig oesophagus/lip

Antibody type	Substrates
Anti-nuclear	Human spleen Rat spleen Rat liver Hep2 cells

Complement indirect IF:

It is a modification of indirect IF for demonstrating complement fixing antibodies in fluids. It may be more sensitive than usual indirect IF in certain instances. A single IgM antibody or two IgG antibodies bound to antigen can generate many molecules of C3. Complement IIF makes use of this amplification principle to increase the sensitivity of the assay system. It attempts to demonstrate the antibody in serum or other tissue fluids. Normal tissue substrate is overlaid with serum or other tissue fluid that has been heated to 56°C for 30 min to destroy complement fixing activity. Antibody against tissue binds during this step. In the second step, the tissue sections are incubated with a source of complement such as fresh human serum.

The complement fixing IgG or IgM antibody that attached to the antigen in the first step can now activate complement in the second step to generate numerous molecules of C3. They are all deposited at the site

of antigen-antibody binding in the tissue. In the third step, sections are incubated with fluorescein labeled antihuman C3 antibodies. They bind to C3 molecules generated in the second step. Sections are washed and examined under fluorescent microscope. In certain situations, few IgG or IgM antibodies bind to tissue antigen that they cannot be detected by classic indirect IF. However, since these molecules generate many C3 complement molecules, complement indirect IF may show fluorescence at the site of antigen antibody binding.

Uses of Immunofluorescence in dermatology for the diagnosis of:

- Pemphigus vulgaris and its variants
- Bullous pemphigoid and its variants
- Pemphigoid (herpes) gestationis
- Epidermolysis bullosa acquisita
- Linear IgA bullous dermatosis (LAD) & CBDC
- Dermatitis herpetiformis
- Discoid lupus erythematosus
- Systemic lupus erythematosus
- Lichen planus
- Porphyria
- Vasculitis
- Amyloidosis

Ideal Site for Taking Skin Biopsy for IF in Various Skin Conditions

Condition	Biopsy site(s)
Pemphigus (all forms)	Perilesional Uninvolved (buttock)
Pemphigoid (all forms)	Perilesional Uninvolved (buttock)
Pemphigoid (herpes) gestationis	Perilesional
Epidermolysis bullosa acquisita	Perilesional
Linear IgA bullous dermatosis (LAD)& CBDC	Perilesional Uninvolved (buttock)
Dermatitis herpetiformis	Uninvolved (buttock)
Discoid lupus erythematosus	Lesional
Systemic lupus erythematosus	Lesional, Sun exposed uninvolved, Non sun exposed uninvolved
Lichen planus	Lesional
Porphyria	Lesional
Vasculitis	Lesional
Amyloidosis	Lesional

Several authors have described the positive effect of saline in increasing the sensitivity of IF analysis. Judd and Lever showed that skin biopsies stored for 24 hours in 0.15 M phosphate buffered saline prior to freezing gave a very high incidence of positive readings in direct IF.[14] It has been suggested that the increase of IF sensitivity by saline incubation is due to improved exposure of epitopes and/or by a decrease of background staining.[15]

Indirect Split Skin Immunofluorescence

This method relies on splitting normal skin through the lamina lucida. Split normal skin is used as a substrate in an indirect IF method to differentiate between antibodies that bind to antigens in the upper lamina lucida or hemidesmosomes and to those that bind to antigens in the lamina densa or sublamina densa

Procedures For Splitting The Skin

1. Incubation with cold 1M NaCl solution
2. Treatment with proteolytic enzymes
3. Induction of suction blister

Direct Split Skin Immunofluorescence

Direct IF is performed on patient's split skin to localise the site of antibody deposition.

Immunofluorescence In Autoimmune Bullous Diseases

The autoimmune bullous diseases result from an immune response against adhesion molecules of the epidermis and basement membrane zone (BMZ).[16]The pemphigus group of diseases is associated with antibodies to desmosomal proteins.[17, 18] The antibodies in each type of pemphigus are directed against a unique desmosomal protein or a specific combination of desmosomal proteins .

Intraepidermal bullous disorders with their target antigen

Pemphigus type	Target desmosomal protein
Pemphigus vulgaris	Desmoglein 3 and desmoglein 1
Pemphigus foliaceous	Desmoglein 1
Paraneoplastic pemphigus	Desmoglein 3, desmoplakin 1, desmoplakin 2, BP 230, envoplakin, periplakin
IgA pemphigus	Desmocollin 1

There is strong direct experimental evidence that antibodies in pemphigus vulgaris (PV) and pemphigus foliaceous (PF) cause acantholysis and blister formation [17, 19, 20] by directly interfering with desmosomal function.[21]

The subepidermal bullous diseases are associated with antibodies against one or more components of the BMZ.[22, 23]

Subepidermal bullous disorder and their target antigen

Bullous disease	Targeted molecule
BP	BP 180, BP 230 (hemidesmosome and lamina lucida)
HG	BP 180, BP 230 (hemidesmosome and lamina lucida)
CP	BP 180, laminin V (hemidesmosome and lamina lucida)
EBA	Type VII collagen (anchoring fibrils)
Bullous SLE	Type VII collagen (anchoring fibrils)
LAD (adults and children)	LAD antigen (BP 180) (hemidesmosome and lamina lucida)
DH	Unknown

The differential diagnosis of a DIF test depends on 4 features:

1. The primary site of immune deposition
2. The class of immunoglobulin or other type of immune deposit
3. The number of immune deposits and, if multiple, the identity of the most intense deposits
4. Deposition in other sites besides the main site.

With above parameters accurate diagnosis can be made in the majority of cases[24]

A. "Intercellular space" deposition

The intercellular space (ICS) fluorescence pattern results from binding of antibodies to desmosomal proteins around the keratinocyte cell surface and is characteristic of the pemphigus group of disorders.

1. IgG deposition in the ICS only

This pattern is characteristic of all types of pemphigus except IgA pemphigus. DIF is positive in 90% to 100% of patients

The pattern of fluorescence appears continuous around individual keratinocytes on scanning magnification and punctate or granular at

higher magnification. The latter pattern reflects binding of antibodies to desmosome associated proteins. Complement component C3 may be seen in a pattern similar to that of IgG.[24]The frequency and, usually, the intensity of C3 deposition are lower than those of IgG.[25]

2. IgG deposition in the ICS and BMZ

The combination of ICS and BMZ deposition may be seen in two settings, PE[26] and PNP.[27]

3. IgA deposition in the ICS

It is characteristic of IgA pemphigus, also known as "subcorneal pustular dermatosis with intercellular IgA deposition" and "intraepidermal neutrophilic dermatosis with intercellular IgA deposition." Because antibodies are directed against desmosomal proteins, the term pemphigus is appropriate for the condition.

BMZ deposition

The detection of immune deposits at the BMZ by DIF is characteristic of the subepidermal bullous diseases. There are several parameters to evaluate for the accurate interpretation of BMZ deposition. These include

1. The type of immune deposit (including class of immunoglobulin)
2. The number of immune deposits, namely, whether the deposition is of one immunoreactant versus multiple immunoreactants
3. The morphology of the fluorescence at the BMZ e.g. continuous, discontinuous, linear, granular, and homogeneous[28]
4. Evaluation for fluorescence in any other site besides the BMZ, such as dermal blood vessels.

Diseases with immune deposits at dermoepidermal junction[29, 30, 31, 32]

- Lupus erythematosus
- Dermatomyositis
- Leucocytoclastic vasculitis
- Bullous pemphigoid
- Herpes gestationis
- Epidermolysis bullosa acquisita
- Dermatitis herpetiformis
- Linear IgA bullous dermatosis

- Porphyria cutanea tarda
- Lichen planus
- Rosacea

There are clues that are helpful in the differential diagnosis. Deposition of C3 with significantly higher intensity than IgG strongly favors the pemphigoid group of diseases (BP, mucosal pemphigoid, and HG). C3 may be the exclusive immunoreactant at the BMZ in patients with HG and occasionally BP. The pattern of deposition in BP and HG has been described as linear, wavy, tubular, and granular. The variation in pattern may result from variations in the angle at which the cryosections are made, the intensity of deposition, and the site of biopsy.[39] In specimens that contain adnexal structures, a similar deposition may be seen along the BMZ of follicular and sweat gland epithelium. Differentiation between BP and HG is not possible by immunofluorescence or histopathology. There is ample evidence confirming that HG is a variant of BP induced by pregnancy.[35]

If the intensity of IgG deposition at the BMZ is significantly higher than that of C3, EBA and bullous SLE are more likely than pemphigoid. The differential expression of intensity between IgG and C3 among the above disorders is not understood.[40]

Deposition of IgG and C3 seen in EBA can be differentiated from BP by salt split skin technique.[41] In BP immunoreactants are deposited in epidermal side alone or both epidermal and dermal side whereas in EBA they are deposited in dermal side. IgG will localize to the roof of the split in the majority of patients, to both roof and floor in 10% but occasionally to the floor alone.[42,43] C3 will always bind to both roof and floor.[44] Deposition in BP is within the lamina lucida. This site of deposition corresponds to the location of the extracellular domain of BP180 antigen that contains the dominant epitopes recognized by the pathogenic BP antibodies.[45] In contrast, deposition of antibodies in EBA is in the sublamina densa area where the target antigen, type VII collagen within the anchoring fibrils, is present.[46] Exclusive deposition on the dermal side may also be seen in antiepiligrin disease, also referred to as antiepiligrin CP.[47]

Pang BK et al has shown that Floor-pattern salt-split skin cannot distinguish bullous pemphigoid from epidermolysis bullosa acquisita.[48]BP can present with a floor pattern fluorescence on salt-split skin. But toad skin can be used to differentiate the two. Only BP will give positive immunofluorescence with toad skin because it has BP and not EBA antigen.

2. Multiple deposits at the BMZ

This pattern of deposition strongly favors EBA and bullous SLE over the pemphigoid group of diseases. In EBA, intense IgG deposition is almost consistently present.[37]The intensity of C3 deposition is usually less than that of IgG. Deposition of IgA is present in approximately two thirds of cases and deposition of IgM in approximately one half of cases.[37]The morphologic pattern of deposition is usually homogeneous, thick, and broad.[49]The BMZ of adnexal epithelia reveals similar deposition.

In bullous SLE, approximately 60% of cases reveal BMZ deposition indistinguishable from that of EBA.[50]In the remaining cases, the deposition is granular and mimics that seen in cases with nonbullous SLE. Compared with nonbullous SLE, bullous SLE is more frequently associated with deposition of IgA.[50] In the absence of clinical history, it is not possible to distinguish EBA, bullous SLE, and nonbullous SLE with certainty. Since most patients with bullous SLE have detectable antibodies to type VII collagen that is also the target of EBA antibodies,[51]differentiation between bullous SLE and EBA is based on an underlying diagnosis of SLE by clinical and serologic criteria.

Revised diagnostic criteria for bullous SLE[52]

1. A diagnosis of SLE based on ARA criteria.
2. Vesicles and bullae arising on, but not limited to, sun exposed skin.
3. Histopathology compatible with DH.
4. Negative or Positive IIF for circulating BMZ antibodies, using separated human skin as substrate.
5. DIF revealing IgG and/or IgM and often IgA at BMZ.

3. Deposition of IgA at BMZ

Linear deposition of IgA at the BMZ is characteristic of LAD[53, 54] The so-called chronic bullous disease of childhood reveals identical findings and represents the childhood form of LAD.[55, 56] Deposition of C3 is present less frequently and with lower intensity compared with IgA.

C. Deposition at the BMZ and blood vessel walls

Homogeneous deposition of immunoreactants (usually multiple) within superficial dermal blood vessel walls, in addition to BMZ deposition, is characteristic of PCT and erythropoietic protoporphyria.[57]

D. Papillary dermal deposition

Granular deposition of IgA and C3 in the papillary dermis and along the BMZ is diagnostic of DH[58]

False-negative DIF in occurs in approximately 10% of specimens[59]and may result from

1. Technical error (e.g., by using wrong or weak antisera)
2. The presence of clinical or subclinical inflammation and early blister formation within the biopsy specimen (this is especially true in cases with PNP)
3. The use of a limited panel of antisera that does not include IgA antisera (for cases with IgA pemphigus).

Imunofluorescence Findings In Various Sub Epidermal Bullous Disorders

Disease	Findings
Bullous pemphigoid	Linear BMZ IgG/C3 ~ 80% IgA/IgM ~ 20%
Cicatricial pemphigoid	Linear BMZ IgG/C3 ~ 80% IgA~ 20%
Epidermolysis bullosa acquisita	Linear BMZ IgG ~ 80% IgA ~ 30%
Linear IgA bullous dermatosis (LAD & CBDC)	Linear BMZ IgA ~ 90% IgG/C3 ~ 20%
Dermatitis herpetiformis	Granular deposits in dermal papillae IgA ~ 100% C3/fibrinogen ~ 40% IgM ~ 10%
Bullous systemic lupus erythematosus	Linear or granular BMZ IgG/C3 IgM ~ 50% ; IgA 60%
Bullous lichen planus	Colloid bodies IgM/IgA/C3 Ragged fibrinogen BMZ band

Colloid bodies can also be seen in bullous pemphigoid. These homogeneous, fibrillar bodies are histologically, immunohistologically and ultra structurally indistinguishable from the colloid bodies found in the lesional skin of lichen planus, lupus erythematosus, dermatomyositis and several other dermatoses.

Bullous Disorders with Negative/Nonspecific IF

Hailey-Hailey disease

Bullous impetigo

Grover's disease

Acantholytic PR

Bullous insect bite

Bullous drug eruption

Drug induced lichenoid photodermatitis

Non-IgA associated vasculitis

AIM OF THE STUDY

1. To find out the incidence of various subepidermal bullous disorders
2. To study the age wise distribution
3. To study the sex distribution
4. To study the disease association
5. To study the immunoreactant pattern of subepidermal blisters on direct immunofluorescence

MATERIALS AND METHODS

In the present study, total number of bullous disorder patients who attended the dermatology OPD, Government General Hospital, Chennai from June 2006 to June 2008 were 107. Of these, 70 patients had intraepidermal blistering disease and 37 patients had various subepidermal bullous disorders. Out of these 37 patients, twenty five cases of subepidermal autoimmune bullous disorders (based on clinical findings) were included in this study.

In all the cases a detailed history was taken. Patients were asked about the duration of the disease, the extent of skin involvement and the presence of oral and other mucous membrane involvement. History of urticarial weals and generalized pruritus was noted. History of drug intake prior to the onset of lesions was taken. Precipitating or aggravating factors were noted. History suggestive of gluten sensitive enteropathy like abdominal pain, diarrhoea and constipation was asked. History of fever, joint pain and photosensitivity was noted. History of blistering on trivial trauma was taken. History of lesions suggestive of lichen planus was also taken. They were enquired about loss of weight, loss of appetite, malena and hemetemesis to rule out internal malignancy. Treatment history, personal history and family history of similar illness was noted.

Each patient was carefully examined .The various clinical parameters studied include:

1. Distribution of vesicles/bullae, number, size, shape, content of bulla: hemorrhagic or clear fluid
2. Presence of bulla on erythematous or non erythematous skin
3. Oral or other mucous membrane involvement
4. Erosions and crusting
5. Hair and nail involvement
6. Palms and soles involvement
7. Nikolsky and Asboe Hansen sign

All the clinical findings were recorded in a standard study proforma. All the patients enrolled in the study were investigated as outlined in the proforma. This study was approved by the Ethical Committee.

Perilesional skin biopsy was performed under strict aseptic precautions, under local anaesthesia using 0.5 ml of 2% lignocaine. A 5 mm disposable punch (Fig.1) was inserted gently into the skin up to the subcutis perpendicular to the skin surface and the biopsy specimen was

gently squeezed out and detached from its socket by sharp scissors. The biopsy specimen was washed in normal saline before putting into the Michel's transport medium (Fig.1). It was labeled and sent to the Department of Dermatology, Kasturba Medical College, Manipal for direct immunofluorescence.

In the laboratory, biopsy specimens were washed in buffer for 30 minutes and then snap frozen. Four micron thick sections were cut in a cryostat (Fig.2), set at -18°C. Sections were washed in PBS (phosphate buffer saline) for 10 minutes and fan dried for another 10 minutes. Sections were then covered with diluted FITC labeled antisera (Fig.3) and then incubated in a moist chamber at 37°C for 30 min. Subsequently specimens were washed with three changes of PBS over a period of 30 minutes. Sections were dried, mounted in buffered glycerol and examined with fluorescent microscope (Fig.4).

All the patients were given appropriate treatment including drugs such as systemic steroids, dapsone, doxycycline and nicotinic acid. Healed lesions were noted for post inflammatory hyper or hypopigmentation or milia formation.

OBSERVATIONS AND RESULTS

Age & Sex Incidence

Of the 25 cases, 13 were males and 12 females. The age of the patient varied from 16 years to 80 years. 18 patients had bullous pemphigoid. Of these 18 BP patients 12 were males and 6 females. Males' age ranged from 16 to 80 years with average age of 47 years. Females' age range varied from 40 to 72 years with average age of 61 years. One patient had linear IgA disease, one patient had Chronic bullous dermatosis of childhood (CBDC), two patients had bullous SLE, one patient had pemphigoid vegetans, one patient had bullous lichen planus and one patient had lichen planus pemphigoides.

Disease wise distribution of cases

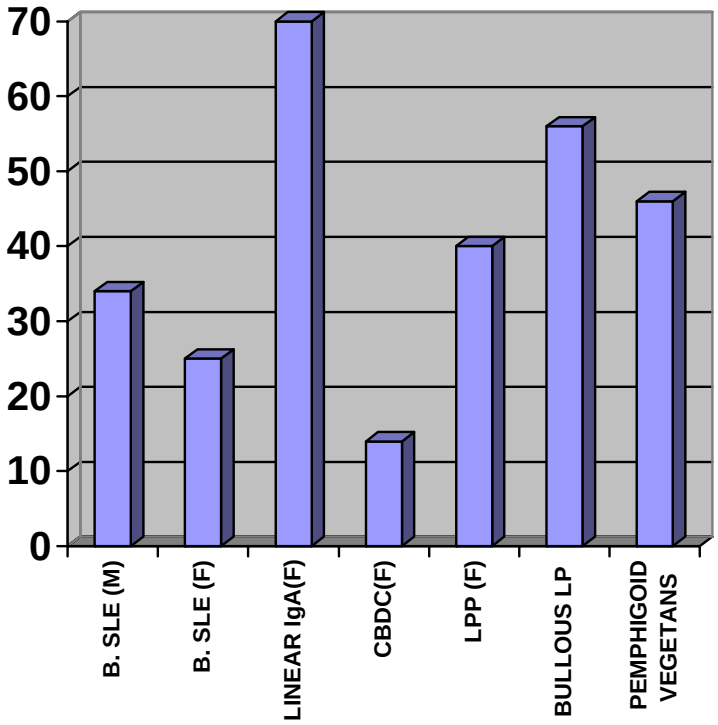
Diagnosis	Total no of cases
Bullous pemphigoid	18
Bullous SLE	2
Linear IgA disease	1
CBDC	1
Bullous LP	1
LP pemphigoides	1
Pemphigoid vegetans	1

Age wise distribution of bullous pemphigoid patients

Age of patient(years)	Bullous pemphigoid	Percentage (%)
0-9	0	0
10-19	2	11.1
20-29	2	11.1
30-39	0	0
40-49	3	16.6
50-59	1	5.5
60-69	6	33.3
70-79	3	16.6
80-89	1	5.5
total	18	100

Sex Wise Distribution of bullous pemphigoid patients

Age of patient(years)	Bullous pemphigoid	
	Male	female
0-9	0	0
10-19	2	0
20-29	2	0
30-39	0	0
40-49	2	1
50-59	1	0
60-69	3	3
70-79	1	2
80-89	1	0
total	12	6



Age wise distribution

Family History

None of the 25 cases gave family history of similar illness in the family.

Clinical Manifestations

The main presenting complaints were blisters and erosions over the skin. Pruritus was the commonest symptom in all the cases. Out of 18 BP patients 12 had prodromal symptoms like itching and urticarial

weals prior to the onset of blisters. Duration between the prodromal symptoms and appearance of blisters varied from 1 month to two years.

Mucous Membrane Involvement

Six cases of bullous pemphigoid had oral mucosal erosion apart from skin lesions. Both the bullous SLE patients had oral erosions.

Cutaneous Manifestations

All cases presented with tense bullae and vesicles, except one patient of pemphigoid vegetans who presented with vegetative plaques. Hemorrhagic bullae were seen in 2 BP patients. Lesions occurred over both normal and erythematous skin in 8 cases and over normal skin alone in rest of the 17 cases. The lesions were distributed over chest, abdomen, back, upper limb, thighs and legs. One case presented with only few lesions localised to hands and abdomen. Typical 'string of pearls' appearance was seen in linear IgA disease and CBDC. In 18 patients lesions resolved with post inflammatory hypopigmentation where as in others it resolved by post inflammatory hyperpigmentation. Nikolsky sign was negative in all the cases. In 7 patients, bulla spreading sign was positive.

Disease Association

Out of 18 bullous pemphigoid patients 10 patients had various associated diseases. 4 patients had diabetes, 5 patients had hypertension, 1 patient had carcinoma prostate, 4 patients had cystic lesions of various organs (kidney, uterus, lung, and liver), 1 patient had renal calculus, and 1 patient had interstitial lung disease.

Investigations

Tzanck smear was negative in all the cases. Five patients had elevated blood sugar. Antinuclear antibody test was positive for SLE patients. Ultrasonography abdomen showed cystic lesions of various organs in four bullous pemphigoid patients. Cysts were seen in lung, liver, uterus and kidney.

Histopathology

Skin biopsy was done in all the cases from an early blister. All cases showed subepidermal bulla. Bulla cavity contained eosinophils and fibrin. In the dermis, inflammatory infiltrate consisted of eosinophils, lymphocytes and few neutrophils. Skin biopsy was also done for other subepidermal bullous disorders and histopathology was characteristic.

Immunofluorescence

Bullous Pemphigoid

All the 18 patients of bullous pemphigoid patients showed C3 deposition at BMZ. C3 was deposited linearly in 17 patients (Fig.5); 4 patients showed strong, 11 patients showed moderately strong and 2 patients showed weak C3 deposition, whereas in one case it was deposited in a weak granular pattern. IgG was present in 11 out of 18 cases i.e. in 61% of cases along with C3 deposition (Fig.6). It was present in a linear fashion in all the cases. Two cases showed IgM deposition (one linear and the other granular pattern) along with IgG and C3. IgA deposition was present in one case of bullous pemphigoid (5.5% of cases) in moderately strong discontinuous pattern. Fibrinogen deposit was present in 3 out of 18 BP cases (16.6% of cases). One case showed colloid bodies with IgM deposition.

LINEAR IgA DISEASE AND CBDC

Linear IgA disease patient showed deposition of IgA at BMZ in linear pattern (Fig.7). In CBDC patient, all the five immunoreactants were present at BMZ i.e. IgA, IgG, IgM, C3 and fibrinogen, but deposition of IgA was stronger as compared to IgG (Fig.8). C3 was present in moderately strong linear fashion.

BULLOUS SLE

One Bullous SLE patient showed moderately strong deposition of IgG in linear pattern, moderately strong deposition of C3 in granular pattern which was present at the floor in the split skin specimen (Fig.9), weak linear deposition of IgM, moderately strong linear deposition of IgA (Fig.10) and fibrinogen at BMZ. Second patient of Bullous SLE showed deposition of IgG and IgM in moderately strong granular pattern (Fig.11), linear weak deposition of C3 and moderately strong deposition of IgA at BMZ. In this patient blood vessel wall deposits composed of fibrinogen were present.

Bullous lichen planus

Bullous lichen planus patient showed deposition of strong cluster of colloid bodies stained with IgM, also few colloid bodies were present which were stained with IgG, C3 and IgA. There was strong ragged fibrinogen band present at BMZ (Fig.12).

Lichen planus pemphigoides

Lichen planus pemphigoides patient showed moderately strong deposition of IgG (Fig.13) and strong deposition of C3 at BMZ. Few

colloid bodies stained with IgM and IgA were also present. Also there was weak deposition of fibrinogen at BMZ.

Pemphigoid vegetans

Pemphigoid vegetans patient showed weak deposition of C3 (Fig.14) and IgG along with focal deposition of IgA and fibrinogen at BMZ.

Treatment

Patients were given appropriate treatment to control the disease.

Course and prognosis

All the cases responded very well to the treatment and showed excellent remission within a month. No mortality was recorded. The disease free interval was variable.

Figure 1 : 5mm disposable punch and michel's transport medium

Figure 2 : Cryostat

Figure 3 : FITC labelled antisera

Figure 4 : Immunofluorescent microscope

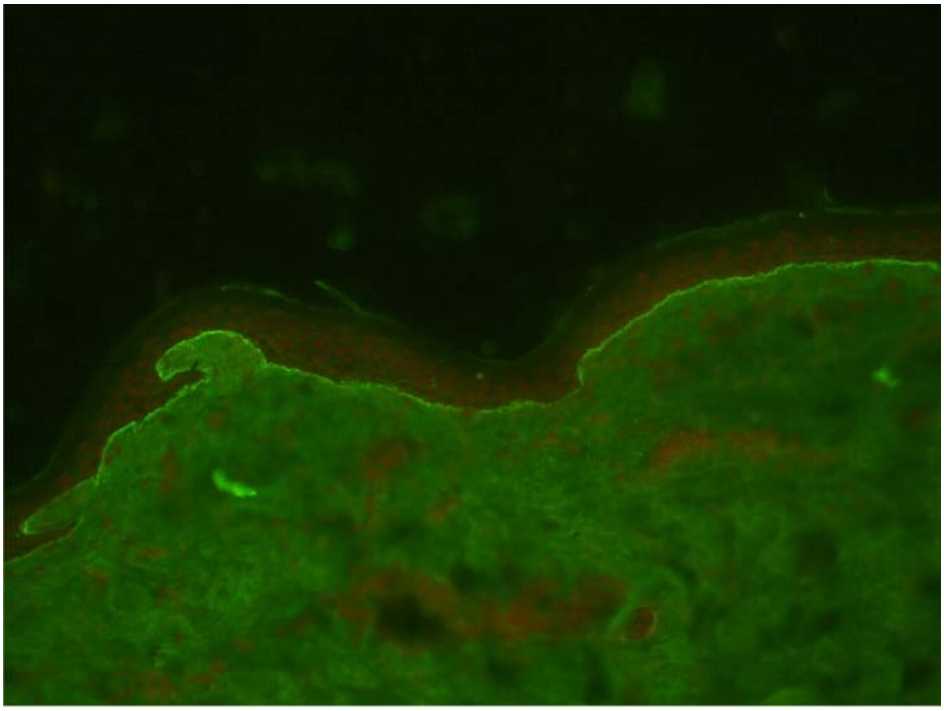


Figure 7 : Linear IgA disease. Linear IgA deposition at BMZ

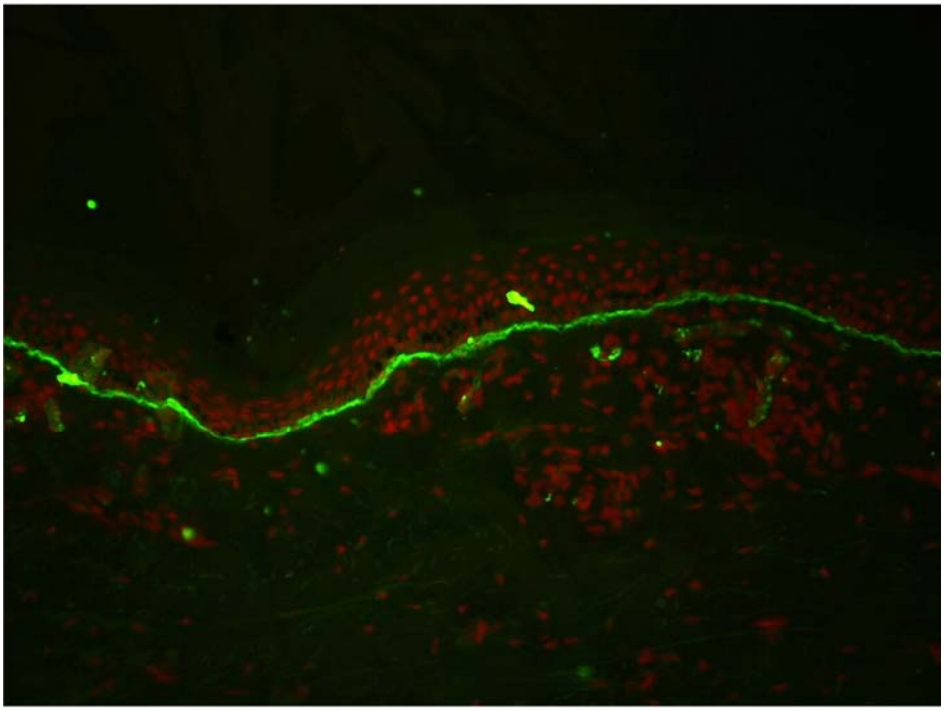


Figure 8 : CBDC. Linear IgA deposition at BMZ

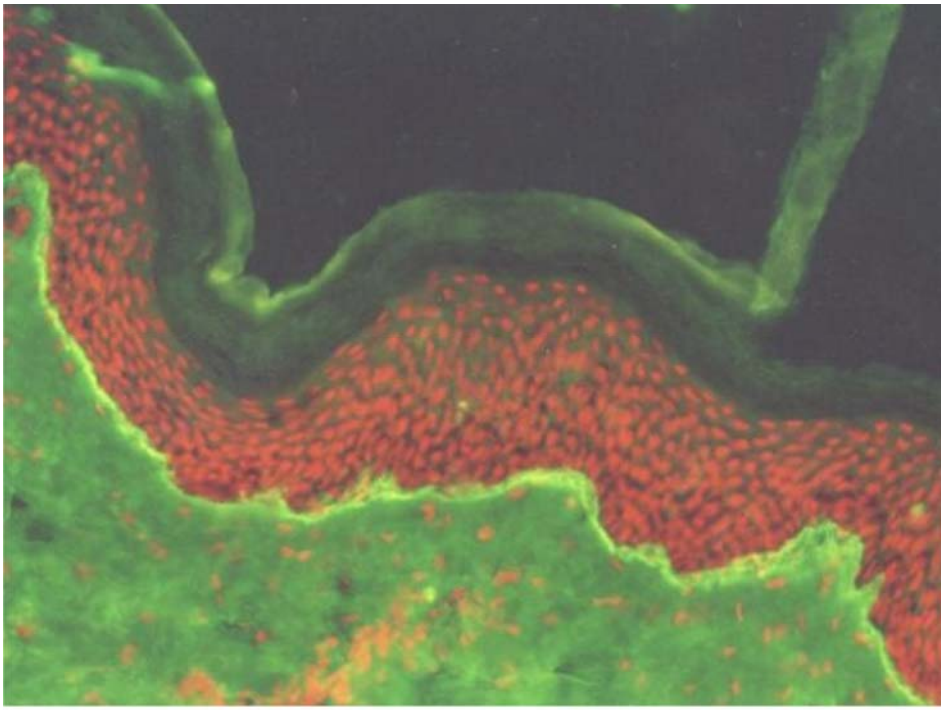


Figure 5 : Bullous pemphigoid. Linear C3 deposition at BMZ

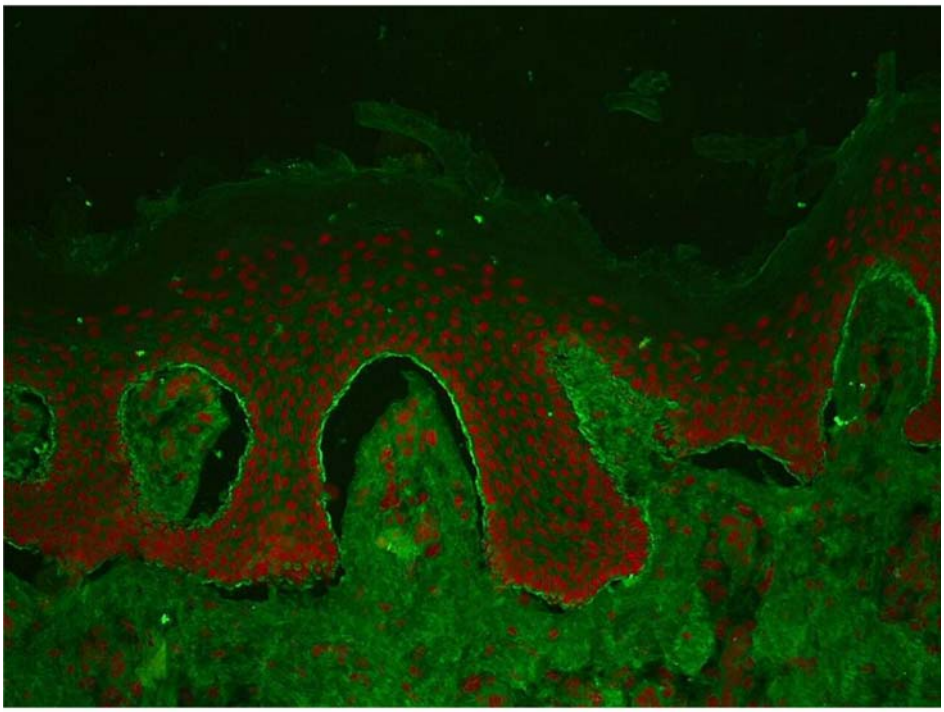


Figure 6 : Bullous pemphigoid. Linear IgG deposition at BMZ

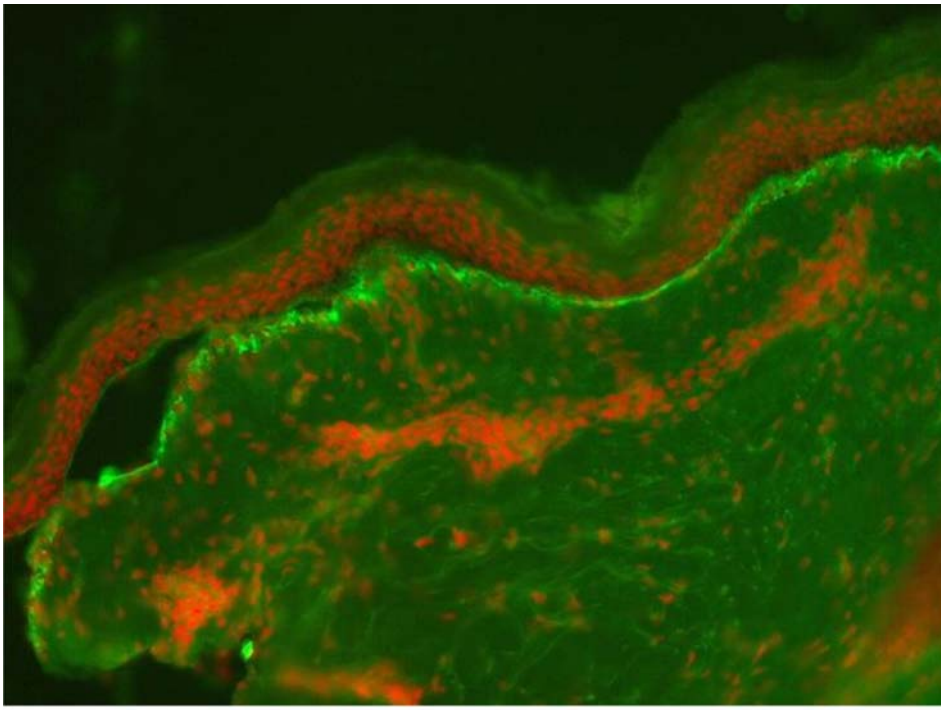


Figure 9 : Bullous SLE. Granular C3 deposition at floor in salt split specimen

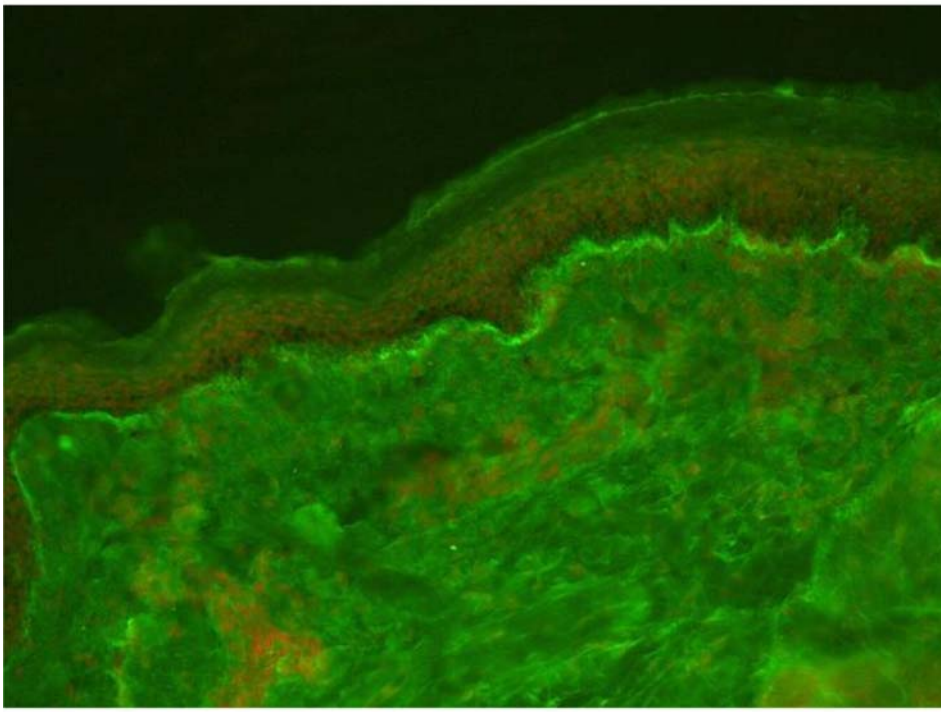


Figure 10 : Bullous SLE. Linear IgA deposition at BMZ

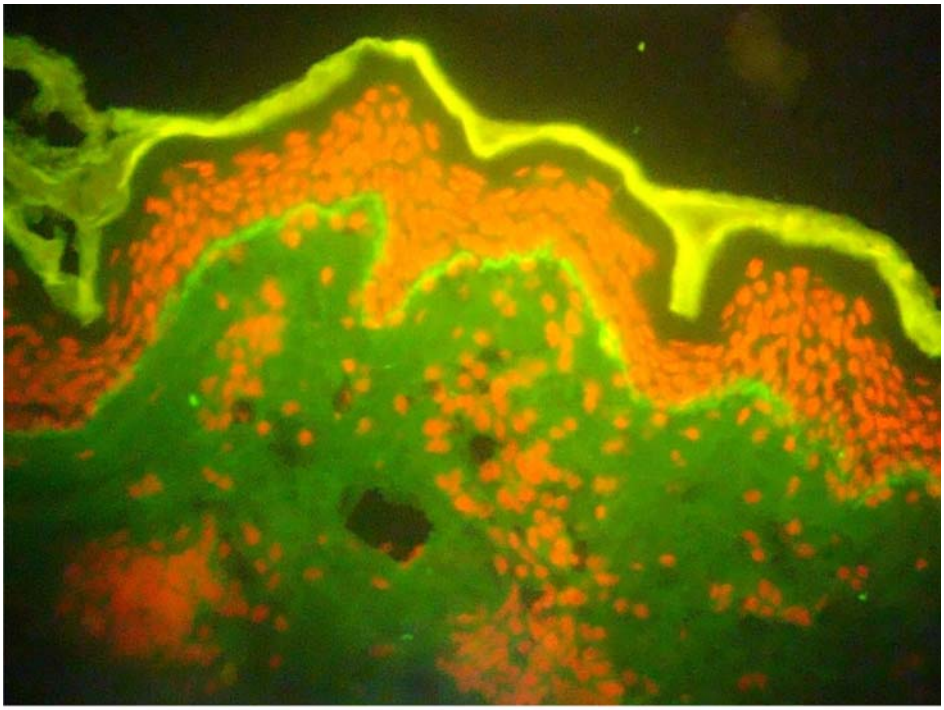


Figure 13 : Lichen planus pemphigoides.
Linear IgG deposition deposition at BMZ

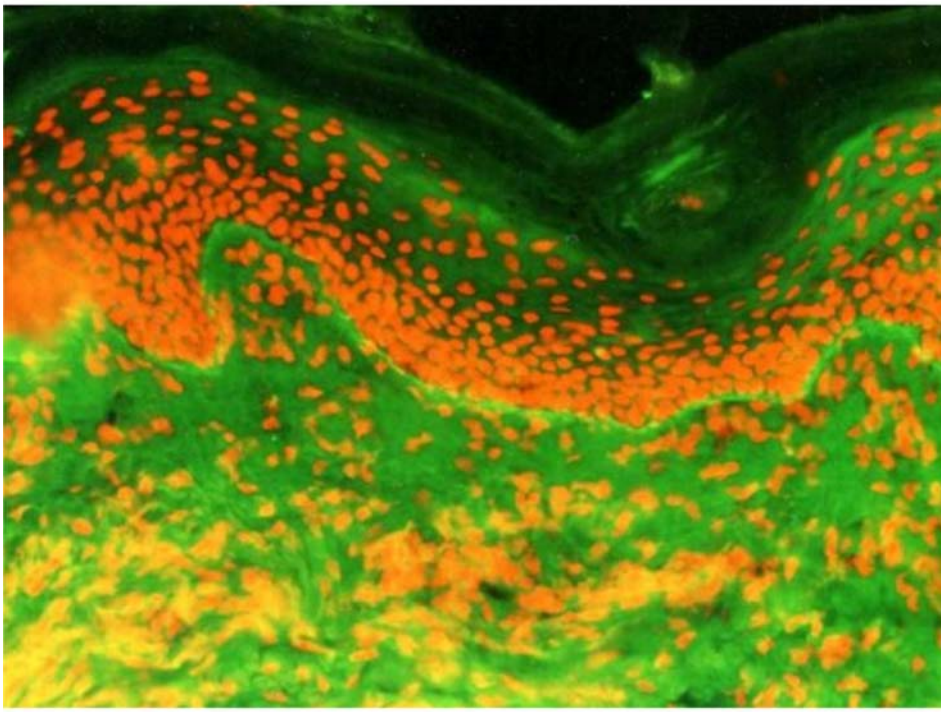


Figure 14 : Pemphigoid vegetans. Linear C3 deposition at BMZ

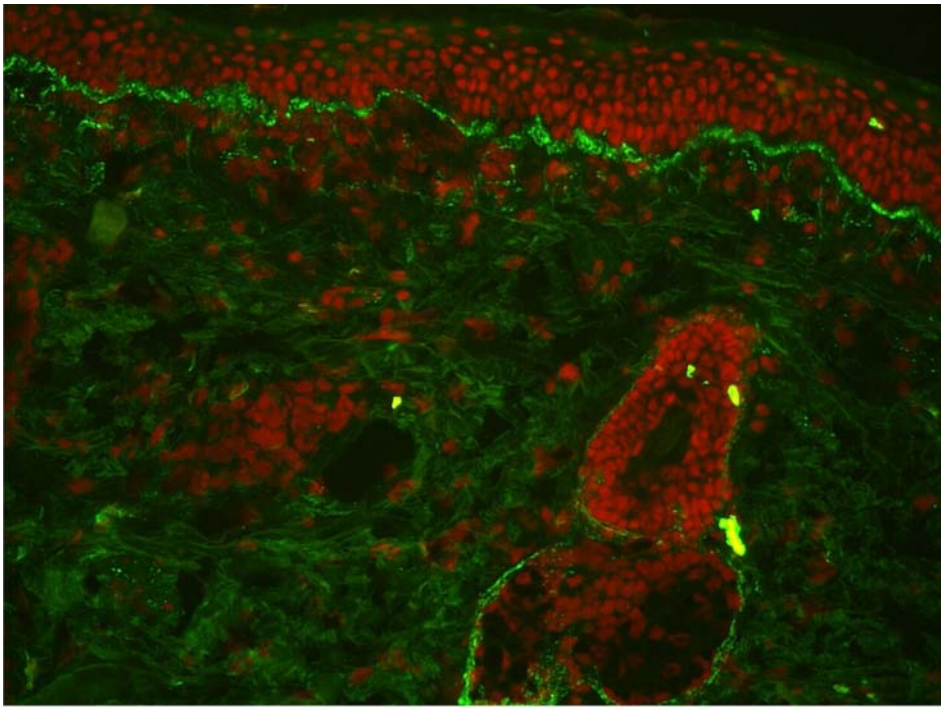


Figure 11 : Bullous SLE. Granular IgM deposition at BMZ

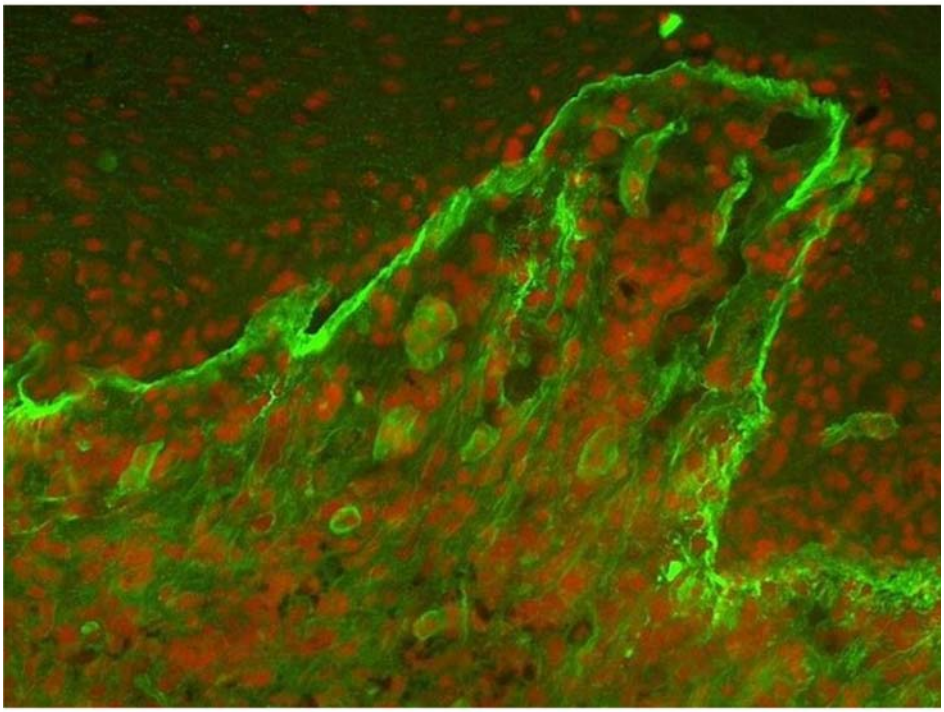


Figure 12 : Bullous LP. Ragged fibrinogen band at BMZ

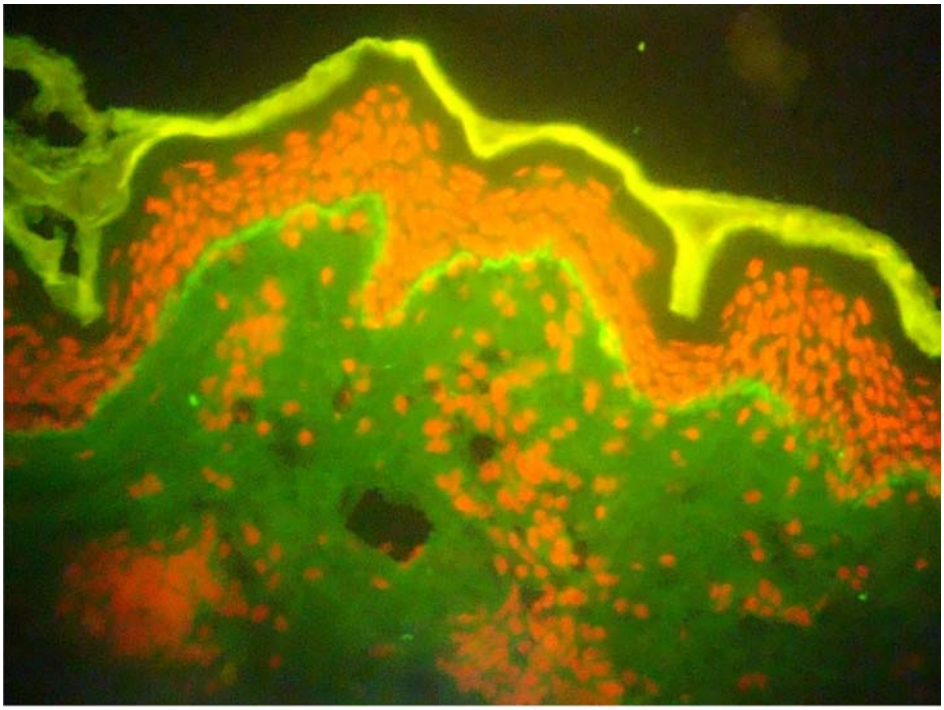


Figure 13 : Lichen planus pemphigoides.
Linear IgG deposition deposition at BMZ

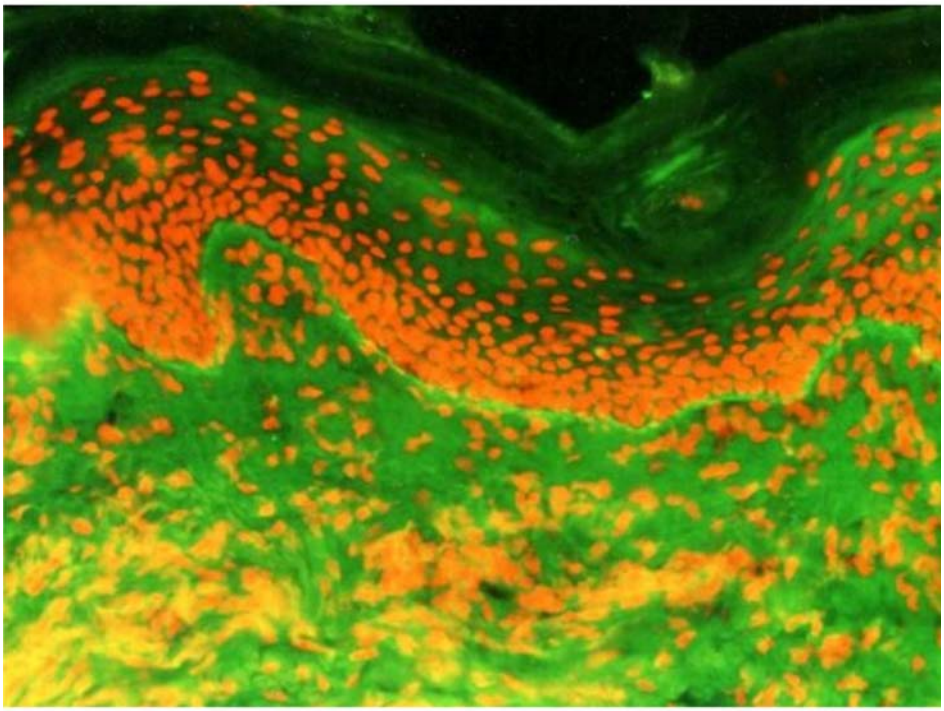


Figure 14 : Pemphigoid vegetans. Linear C3 deposition at BMZ

DISCUSSION

Pemphigus vulgaris has been reported to be the most common autoimmune blistering disease in eastern countries, such as Malaysia, China and India. Whereas in Western Europe, bullous pemphigoid is the most common immunobullous disorder. During the study period bullous pemphigoid was the commonest subepidermal autoimmune blistering disorder .It constituted 30 out of 107 cases (28%). But overall pemphigus vulgaris was the commonest blistering disorder (70 out of 107 cases).

The mean age of onset of bullous pemphigoid was 56 years in males. The age range varied from 16 years to 80 years. Whereas in females age range varied from 40 years to 70 years with a mean age of 61 years. Although the age range for female patients was narrow, they had higher mean average age of presentation of disease. This is consistent with other studies by lever W F[60] and Amos B et al.[61]

The youngest patient was 16 year male in our study. Although bullous pemphigoid is a disease of the elderly, it can occur in individuals less than 40 years. Several cases have been described in children. Few cases has also been reported in infants by Oranje AP and Amos B et al.[61]

Out of 30 bullous pemphigoid patients 20 were males and 10 were females, constituting male to female ratio of 2:1. Incidence of bullous pemphigoid is more in males as compared to females and similar studies were reported by Jung M et al.[62]

Out of 18 BP patients 6 had oral lesions (33%).According to the literature oral lesion can occur in 10-30 % of patients. Similar studies has been reported by Person JR et al.[63]

Bullous pemphigoid can be associated with malignancy. Although in many studies there is no increase in incidence of malignancy in bullous pemphigoid patients, gastric carcinoma is the most common malignancy reported by Ogawa H et al [64] in their series of more than 1000 BP patients. In our study one patient had prostate carcinoma. This rare association of prostate adenocarcinoma with bullous pemphigoid has been reported by Oztürkcan S et al.[65]

Four out of 18 patients (22%) of bullous pemphigoid showed cystic lesion of various organs including liver, kidney, lung and uterus. Previously no such association has been described in the literature.

DIF

C3 deposition was seen in 100% of BP cases, linear deposition in 17 out of 18 patients and weak granular fashion in 1 patient. IgG deposition was seen in 66% of patients.ie 12 out of 18 patients, linear in 4 cases and discontinuous in 8 patients. Chan YC et al have shown in their study linear deposits of IgG and C3 along the dermoepidermal junction in twenty-one of 23 (91%) patients.[66] Similar results have been reported by Chang YT et al in their study of 88 patients.[67] Harrist TJ and Mihm MC have shown that C3 is present in 100% and IgG in 65% to 95% of patients.

Positive deposition of C3 but negative staining for IgG on direct immunofluorescence (DIF) studies has been noted in some patients.[68] Following factors contribute to false-negative staining for IgG on DIF in some BP patients:

1. Sub threshold IgG in skin specimens
2. Limited reactivity of commercial antihuman IgG conjugates to the IgG4 subclass
3. Decreased sensitivity of DIF compared with double antibody methods for the detection of IgG.

The use of sandwich double antibody immunofluorescence methods to test for IgG and/or IgG subclasses may be helpful in definitively diagnosing BP in patients with negative IgG and positive C3 staining on DIF[68]

One patient of bullous pemphigoid showed colloid bodies stained by IgM. Similar colloid bodies has been described by Y. Horiguchi et al in 8 out of 18 patients in their studies.[69] These homogeneous, fibrillar bodies were histologically, immunohistologically and ultrastructurally indistinguishable from the colloid bodies found in lesional skins of lichen planus, lupus erythematosus, dermatomyositis and several other dermatoses.[69] In BP, degenerated keratinocytes adjacent to the blister roof, may, after undergoing a filamentous change, drop off into the dermis and subsequently form homogeneous, fibrillar bodies in the uppermost dermis when re epithelization is completed. Some findings that may help favor lichen planus include the tendency for cytoid bodies in LP to cluster in groups, to be present in high number, to be larger, to have higher fluorescent intensity, and to contain multiple immune deposits.[70]

Two cases of BP also showed weak deposition of IgM along with IgG and C3.Fibrinogen deposition was seen in 3 patients of BP apart

from other immunoglobulin and C3. Similar results have been shown by Ahmed AR et al, [71] and Cormane RH.[72] They have shown the deposition of IgM, IgA, IgD and IgE at the dermoepidermal junction. This is also consistent with the findings of Provost TT and Tomasi TB who have shown that IgE, alternate pathway components and fibrinogen can be deposited along DEJ in bullous pemphigoid patients.[73]

Linear IgA disease patient showed deposition of only IgA at BMZ in linear pattern. This is consistent with the diagnosis. Studies by Wojnarowska F, Bhogal B and Black MM have shown deposition of other immunoreactants also (IgM, IgG and C3) in 20-30% of patients.[74,75]

In CBDC patient all the five components were present i.e. IgA, IgG, IgM, C3 and fibrinogen, but deposition of IgA was stronger as compared to IgG. C3 was present in moderately strong linear fashion which is consistent with the diagnosis of CBDC. According to Petersen MJ et al that if both IgA and IgG are present and deposition of IgA is more intense than IgG, and C3 deposition is strong, than it should be considered as a case of linear IgA dermatosis.[76] There have been many case reports in which there is deposition of only IgG and IgA. These cases should be considered as subset of linear IgA disease. Metz BJ et

al[77] has described a patient of Linear IgA dermatosis with IgA and IgG deposition only at BMZ. Similar deposition has been described by Watanabe M et al[78] and Kersting E et al.[79]

Bullous SLE patient showed moderately strong deposition of IgG in linear pattern, moderately strong deposition of C3 in granular pattern which was present at the floor in the split skin specimen, deposition of IgM in weak linear, and IgA and fibrinogen were present in moderately strong linear pattern. Another patient of Bullous SLE showed deposition of IgG and IgM in moderately strong granular pattern, weak deposition of C3 and moderately strong deposition of IgA.

Similar results have been reported by Gammon WR et al in their studies.[80]They have shown that IgG is always present and IgA and IgM are also frequently deposited at the BMZ. The pattern of deposition may be granular (60%), linear (40%) or rarely fibrillar.[80] A linear rather than granular pattern along the BMZ is associated with the presence of higher titer of circulating autoantibodies.[80]

Both of the Bullous SLE patients showed deposition of IgA. Gammon WR et al have shown that Bullous SLE is associated with a higher incidence of IgA deposition (76%) than other forms of SLE (17%) and this may also correlate with renal involvement.[81]

Lichen planus pemphigoides patient showed moderately strong deposition of IgG and strong deposition of C3 at BMZ. Few colloid bodies stained with IgM and IgA were also present in the superficial dermis. There was also weak deposition of fibrinogen at BMZ.

Lichen planus pemphigoides is a rare clinical variant of bullous pemphigoid and DIF findings are similar to BP. Okochi H et al have drawn conclusion from their study that the presence of C3 alone or with IgG along the dermoepidermal junction is confirmatory on DIF[82] Vibhu Mendiratta et al have shown deposition of IgG and C3 at BMZ in lichen planus pemphigoides patient.[83]

Bullous LP patients showed strong cluster of IgM colloid bodies in superficial dermis. There were also colloid bodies with weak deposition of IgG, C3 and IgA. Also there was strong deposition of ragged fibrinogen band along DEJ. A Sandra et al have shown in their study of 18 bullous lichen planus patients similar findings.[84] In their study ragged fibrinogen band was present in all the patients whereas colloid bodies demonstrating IgG, C3, IgM and IgA were seen in 88% of patients. In bullous lichen planus patients immune deposits are present within cytoid bodies in the superficial dermis, as well as along the DEJ.[85] The most frequently present immune deposits are IgM and

fibrinogen.[85, 86] Deposition of IgG, IgA, and C3 is less frequently present.[87, 88]

Gawkrodger DJ et al compared two patients with lichen planus pemphigoides and two with bullous lichen planus and showed direct immunofluorescence was positive in lichen planus pemphigoides and negative in bullous lichen planus.[89]

Pemphigoid vegetans patient showed C3 and IgG deposition with weak deposition of IgA and fibrinogen. Chan LS et al described a patient of pemphigoid vegetans with deposition IgG at BMZ.[90] They considered it to be a variant of bullous pemphigoid. Marie Ogasawara et al described a patient of pemphigoid vegetans with IgA, IgG and weak C3 BMZ deposition.[91] Lawrence S Chan et al reported a patient of pemphigoid vegetans with linear IgG deposition at BMZ.[92] IgG, IgM, C3 and fibrin deposition has also been described by Winkelmann RK and Su WPD who first described this clinical entity.[93] Y. Ueda et al considered it to be a variant of bullous pemphigoid.[94]

CONCLUSION

1. Bullous pemphigoid was the most common subepidermal blistering disease.
2. Bullous pemphigoid was twice as common in males as in females.
3. Rare association of cystic disease of various organs was noted with bullous pemphigoid group of patients.
4. Linear pattern deposition of C3 at BMZ was seen in 17 patients and granular pattern in one patient.
5. Colloid bodies were also seen in one BP patient, they are not specific for any disease.
6. Deposition of IgM and fibrinogen, apart from other immunoreactants were seen in bullous pemphigoid patient.
7. Linear IgA patient showed linear IgA deposition at BMZ.
8. In CBDC patient IgA, IgG, IgM, C3 and fibrinogen deposition was seen.
9. Multiple immunoreactant deposition was seen in bullous SLE patient.

10. In bullous SLE, immunoreactant deposition was seen in both granular and linear patterns.

11. Lichen planus pemphigoides patient showed IgG and C3 deposition at BMZ and few colloid bodies stained with IgM and IgA.

12. Bullous lichen planus patient showed colloid bodies stained by IgM, IgG, IgA and C3. Strong ragged fibrinogen band was seen at BMZ.

13. Pemphigoid vegetans patient showed C3, IgG, IgA and fibrinogen deposition at BMZ.

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PROFORMA

Name : Age : Sex :

Address : Occupation :

Chief Complaints :

A) Skin blisters

B) Oral lesions

History of presenting illness :

History of urticarial weals and generalized pruritus

History of drug intake prior to the onset of lesions

Precipitating or aggravating factors

History of abdominal pain, diarrhoea and constipation

History of fever, joint pain and photosensitivity

History of blistering on trivial trauma.

Loss of weight, loss of appetite, malena and hemetemesis

Treatment history

Personal history

Family history

General examination :

Pallor/icterus/cyanosis/clubbing/lymphadenopathy/edema

Vital signs: BP:

PR

RR:

Systemic examination

RS

CVS

P/A

CNS

Dermatological examination :

Vesicle/ bulla

Distribution of vesicles/bullae, number, size, shape, content of bulla: haemorrhagic or clear fluid

Presence of bulla on erythematous or non erythematous skin

Oral or other mucous membrane involvement

Palms and soles involvement

Erosions and crusting

Hair and nail involvement

Nikolsky and Asboe Hansen sign

Investigations

Hemogram

LFT

RFT

Urine albumin/ sugar

Blood sugar

Tzanck smear

Skin biopsy

Direct immunofluorescence

Diagnosis